Intrafamily and Interfamilial Phenotype Variation and Immature Immunity in Patients With Netherton Syndrome and Finnish SPINK5 Founder Mutation

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**IMPORANCE** Netherton syndrome (NS) is a rare and severe genodermatosis caused by SPINK5 mutations leading to the loss of lymphoepithelial Kazal-type–related inhibitor (LEKTI). Netherton syndrome is characterized by neonatal scaling erythroderma, a bamboo-like hair defect, a substantial skin barrier defect, and a profound atopic diathesis. Netherton syndrome has been proposed to be a primary immunodeficiency syndrome because of the high frequency of infections. The precise mechanisms underlying the disease are not fully understood.

**OBJECTIVE** To study the association of the SPINK5 mutation with the NS phenotype and the extent of immunologic deficiencies in NS.

**DESIGN, SETTING, AND PARTICIPANTS** Relevant tissue samples and follow-up data from 11 patients with NS from 7 families, including 3 multiplex families, were collected, constituting all known patients with NS in Finland. Another patient with NS from a neighboring country was included. Data were collected from August 10, 2011, to February 20, 2015. SPINK5 mutations were sequenced, and thorough clinical evaluation and histopathologic and immunohistochemical evaluations of skin samples were performed. The function of natural killer cells, lymphocyte phenotype, and serum immunoglobulin subclass levels were evaluated. Data analysis was conducted from October 19, 2011, to February 20, 2015.

**MAIN OUTCOMES AND MEASURES** The nature of SPINK5 mutations and their correlation with phenotypes in Finnish patients with NS, intrafamilial phenotype variations, and the type of immunologic defects in NS were evaluated.

**RESULTS** Among the 11 Finnish patients with NS (8 male [73%]; 3 female [27%]; mean [SD] age, 30.1 [9.1] years), a Finnish founder mutation c.652C>T (p.Arg218*) in SPINK5 was identified in 10 patients from 6 families who all originated from the same region. Eight patients were homozygotes for this mutation and 2 siblings were compound heterozygotes with a splice site mutation c.1220 + 1G>C (IVS13 + 1G>C). Phenotypes were comparable, but some intrafamilial and interfamilial variations were noted. Compound heterozygous patients had a milder phenotype and showed residual LEKTI expression. A previously unreported c.1772delT (p.Leu591Glnfs124*) mutation was found in 1 patient with a phenotype similar to the patients homozygous for the founder mutation. The patient from the neighboring country had a distinct phenotype and different mutations. Immunologically, natural killer cells had an immature phenotype and impaired cytotoxicity and degranulation, levels of memory B cells were reduced, and serum IgG4 levels were elevated. Intravenous immunoglobulin treatment has been beneficial in 1 patient with NS.

**CONCLUSIONS AND RELEVANCE** This report discloses a prevalent SPINK5 founder mutation in Finland and illustrates NS phenotype variability. Our results also point to a possible role of immature immunity in the frequent infections seen in NS.

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Netherton syndrome (NS) is a rare and severe autosomal recessive ichthyosiform skin disease caused by mutations in the SPINK5 (serine protease inhibitor Kazal type 5) (HGNC 15464) gene. SPINK5 encodes LEKTI (lymphoepithelial Kazal-type-related inhibitor), a serine protease inhibitor expressed in the upper epidermal layers of the skin and in stratified epithelia. Defective LEKTI expression leads to congenital extensive skin inflammation and scaling, a bambooleike hair defect (trichorhhexis invaginata), and multiple atopic manifestations. Patients with NS typically develop at least 1 of the following symptoms: pruritic atopic dermatitis-like skin lesions, allergic asthma, urticaria, angioedema, allergic rhinitis, and food allergies. Patients with NS often display recurrent bacterial infections, hypernatremic dehydration due to transepidermal water loss, failure to thrive, recurrent bacterial infections, hypernatremic dehydration, and elevated levels of serum total IgE (sometimes >10,000 kU/l [to convert to micrograms per liter, multiply by 0.0024]), and blood hypereosinophilia.

Murine models of NS and human studies have shown that the loss of LEKTI causes overactivity of tissue kallikreins (KLKs), especially KLK5 and KLK7. This overactivity leads to stratum corneum detachment through degradation of desmoglein 1 (Dsg-1), skin inflammation, and elastase 2–induced profilaggrin degradation, all of which result in a defective skin barrier. In addition, KLK5 induces the production of the major pro-helper T cell 2 (Th2) cytokine thymic stromal lymphopoietin. Various immunologic defects have been reported to contribute to increased susceptibility to infections, but the exact nature of these defects and the underlying mechanism for hyper-IgE levels and atopic manifestations are not fully understood.

We describe herein a Finnish cohort of 11 patients originating from 7 families and a 12th patient from a neighboring country. We identified a SPINK5 founder mutation in 10 patients originating from the same geographic part of Finland. Seven patients were born to 3 families and thus, intrafamily and interfAMILY phenotype differences against the same genotype background were studied. In 7 of the patients with NS, the natural killer (NK) cells had an immature immunophenotype, with impaired cytotoxic and degranulation responses, and IgG4 levels were elevated in 4 of 5 patients. Intravenous immunoglobulin (IVIG) therapy proved beneficial in 1 patient with severe disease.

Methods

Patients

We recruited patients with NS from the Helsinki and Tampere University hospitals. All patients underwent clinical evaluation by at least 2 of us (including K.H.-J., S.-L.L., M.T., M.-L.T., and H.H.), and additional data were collected from patient records. Data were collected from August 10, 2011, to October 6, 2015. This study was approved by the Coordinating Ethical Review Board of the Helsinki and Uusimaa Hospital District of Finland and conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all patients and/or their parents.

SPINK5 Mutation and Haplotype Analysis

We extracted DNA from blood samples using standard protocols. SPINK5 mutations of families I, II, and VIII were analyzed at the Department of Genetics, Necker Hospital for Sick Children; those from families III through VII, by GeneDx. Haplotype analysis with 5 polymorphic microsatellites flanking SPINK5 was performed for patients and parents from families I through VI (eTable 1 in the Supplement).

Reverse Transcription–Polymerase Chain Reaction Analysis and Complementary DNA Sequencing

We extracted RNA from keratinocytes, and first-strand complementary DNA synthesis was performed using reverse transcriptase (M-MLV; Invitrogen). Sequences of primers used were ACCCTATTCTGGTGCAGAT (exon 11) and CTCTTCTCTTTCCGTGA (exon 16). Complementary DNA amplimers were cloned into a plasmid vector (pGEM-T; Promega) and sequenced using T7 and sp6 primers. The genomic environment of the mutation and exonic splicing sequences were analyzed with the Automated Splice Site Analysis (https://splice.cmh.edu/).

FLG Genotyping

Genomic DNA from all patients was genotyped for prevalent FLG (HGNC 3748) null mutations in Europe. Mutations R501X and R2447X were analyzed by Taqman allelic discrimination (ABI 7500; Applied Biosystems). Deletions 2282del4 and 3702delG were typed by sizing fluorescently labeled polymerase chain reaction products on a sequencer (ABI 3130; Applied Biosystems).

Histopathologic and Immunohistochemical Analysis

Skin biopsies from patients I:1 to VIII:1 were processed and analyzed with standard procedures by an experienced dermatopathologist (L.J.). Expression of LEKTI, filaggrin, and Dsg-1 was studied by immunohistochemistry using antibody L8016 (clone 6A10B, diluted 1:1000; US Biological) and a visualization kit (EnVision; Dako). Filaggrin expression was studied likewise with antibody filaggrin-NCL (clone 15C10, pretreated in Tris-EDTA buffer [pH, 9.0], diluted 1:50; Novocastra Laboratories); Dsg-1 expression, with a monoclonal anti-Dsg-1 antibody (monoclonal antibody to Dsg-1, clone Dsg-1-P23, dilution 1:1000; Progen Biotechnik GmbH). For Dsg-1, antigen retrieval was performed with microwave treatment, and the bound antibody was visualized with a peroxidase reagent kit (peroxidase universal anti–mouse-rabbit Ig MP-7500; ImmPRESS; Vector Laboratories).

Immunologic Studies

Complete and differential white blood cell counts, lymphocyte subsets (T, B, NK, and regulatory T cells), and vaccine responses were determined according to routine and accredited laboratory methods (http://www.huslab.fi and http://www.fimlab.fi). We collected fresh heparin blood samples from 7 patients with NS (I:1, II:1, V:1, V:2, VI:1, VII:1, and VIII:1) and 6 healthy adult control individuals. Owing to ethical considerations, blood samples from healthy, age- and sex-matched children were not available. The NK cell pheno-
type was studied with multicolor flow cytometry using anti-CD45, anti-CD3, anti-CD14, anti-CD19, anti-CD57, anti-CD62L, anti-CD27, and anti-CD45RA antibodies.17

Cytotoxicity and Degranulation and Cytokine Assays
Mononuclear cells were isolated from peripheral blood using density gradient centrifugation (Ficoll-Paque; GE Healthcare). Cytotoxicity of NK cells against K562 cells was studied with mononuclear cells or purified NK cells as described previously.17 To examine the degranulation capacity of NK cells, mononuclear cells were stimulated with K562 cells, and the expression of CD107a/b was measured by flow cytometry.17 To study the cytokine secretion capability of NK cells, mononuclear cells were stimulated with phorbol myristate acetate and calcium 1, and cytokine (tumor necrosis factor and interleukin-12) production was monitored.17

Statistical Analysis
Data were analyzed from October 19, 2011, to February 20, 2015. All statistical analyses were performed with GraphPad Prism software (GraphPad Software Inc.). We used the nonparametric Mann-Whitney test for comparison between 2 groups and 2-way analysis of variance for comparison in the cytotoxicity assay. P < .05 was considered statistically significant.

Results
A SPINK5 Finnish Founder Mutation in Exon 8 and a Novel Mutation in Exon 19
We included 11 Finnish patients and a 12th patient from a neighboring country (8 male [67%]; 4 female [33%]; mean [SD] age, 9.6 [4.2] years). Affected individuals from families I to V were all homozygous for the same nonsense mutation c.652C>T p.(Arg218*) in exon 8 of SPINK5 (Table). This mutation predicts a premature termination codon, which results in a missing or a truncated protein synthesis. All parents from these 5 families originated from the same western region of Finland. Haplotype analysis in families I to VI revealed that the c.652C>T mutation segregated with the same combination of alleles, supporting a founder effect in these families (eTable 2 in the Supplement). The c.652C>T mutation has been reported previously only in a single compound heterozygote Finnish-Italian patient.18 Results of immunohistochemical analysis for LEKTI were negative (eFigure 1A in the Supplement).

Siblings VI:1 and VI:2 were compound heterozygotes for the founder mutation c.652C>T (maternally inherited) and a paternal inherited nucleotide substitution c.1220 + 1G>C (IVS13 + 1 G>C) in intron 13 (Table), predicted to cause abnormal pre-messenger RNA splicing.19 Reverse transcription-polymerase chain reaction analysis from keratinocytes of case VI:1 revealed 3 different-sized amplimers. Sequencing of these products showed removal of the last 15 nucleotides of exon 13 or frame-shifts leading to a premature termination codon (eFigure 2 in the Supplement). Expression of LEKTI was weakly positive in the upper parts of the epidermis of heterozygote patient VI:1 (c.652C>T, c.1220 + 1 G>C) in a spotty pattern around hair follicles and eccrine ducts (eFigure 1B and eTable 3 in the Supplement).

Patient VII:1 was homozygous for a previously unreported SPINK5 single-nucleotide deletion c.1772delT p.(Leu591Glnfs*124) in exon19. This mutation is expected to cause a reading frame shift predicting a premature termination codon 123 codons downstream of the mutation, resulting in a severely truncated or missing LEKTI protein. The parents of patient VII:1 have common ancestors 4 and 5 generations back.

Patient VIII:1 was a compound heterozygote for the following 2 nonsense mutations predicting premature termination codons: c.1048C>T p.(Arg350*) and c.2098G>T p.(Gly700*) in exons 12 and 22, respectively (Table). The parents of patient VIII:1 are from a neighboring country. These mutations were previously reported in patients of unspecified geographic origin.14,20

None of the most frequent European recurrent FLG mutations was identified in the patients with NS. Filaggrin expression was normal (eFigure 1D in the Supplement) in only a few patients, but it was decreased or detected in a spotty or linear fashion in most of the patients (eFigure 1E in the Supplement). Expression of Dsg-1 was most often reduced in the stratum spinosum in a spotty pattern in the upper layers when the stratum granulosum was absent or reduced (eFigure 1G and H and eTable 3 in the Supplement).

Skin Phenotype and Recurrent Infections Associated With Homozygous SPINK5 Founder Mutation c.652C>T
Seven of 8 patients had neonatal scaling erythroderma requiring hospitalization (Table and Figure 1), and 5 of 8 patients had hypernatremia. At 3 postnatal days, all had developed scaling erythroderma. Ichthyosis linearis circumflexa developed in all patients from 6 months to 8 years of age. With advanced age, the skin has improved significantly or at least slightly with occasional flares in all patients (Table). All 8 patients had severe and continuous pruritus, felt constantly cold, and had total anhidrosis.

Recurrent skin infections and conjunctivitis were common, especially during the first 3 years of life (Table). Increased skin exfoliation has caused recurrent external otitis in most (Table), requiring regular rinsing and temporary topical and systemic antibiotics. All patients had multiple atopic manifestations (Table) described in detail elsewhere.21 Basic skin treatment consisted of topical emollients, corticosteroids, and antibacterial creams when needed. Patient III:1 received acitretin for a few months with no benefit. Patient II:1 has received IVIG at 385 mg/kg per month for 11 months now. Rapid improvement within weeks occurred with decreased pruritus and reduced erythema and skin flaring. No skin, external ear, or eye infections have occurred during IVIG treatment. In addition, tolerance against many allergens has increased, which allowed expansion of his diet. No significant changes in blood eosinophil or IgG4 levels have occurred, but IgE levels have slightly declined from 7935 to 6221 kU/l.

A Milder Phenotype in the Compound Heterozygote Patients and Intrafamilial Variation
Skin symptoms at birth were missing or only patchy in affected siblings VI:1 and VI:2 (Table). Later erythematous flares...
<table>
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<tr>
<th>Family No.:Patient No. by SPINK5 Mutation</th>
<th>LEKTI expression</th>
<th>Age</th>
<th>Allergy symptoms, U/AO/AS/R</th>
<th>IgE level, kU/L</th>
<th>Gestation, mean (SD), wk</th>
<th>Neonatal hospitalization</th>
<th>Hypermatura</th>
<th>Skin condition at birth/at 3 d</th>
<th>Skin condition improvement</th>
<th>Recurrent skin infections</th>
<th>Prolonged antibiotics</th>
<th>External otitis</th>
<th>Conjunctivitis</th>
<th>Sweating/pruritus</th>
<th>ILC</th>
<th>Brittle eyebrows/ eyelashes</th>
<th>Ectropium</th>
<th>Nail abnormalities</th>
<th>Susceptible to UV burns</th>
<th>GER/diarrhea</th>
<th>Growth restriction height SD (age, y)</th>
<th>Latest height SD</th>
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<tr>
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<td>−</td>
<td>−</td>
<td>−</td>
<td>1518</td>
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<td>−</td>
<td>SE/SE</td>
<td>PE</td>
<td>a,b,c,d,e,h,i</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
<td>17433</td>
<td>36 (5)</td>
<td>+</td>
<td>+</td>
<td>SE/SE</td>
<td>PE</td>
<td>a,b,c,f,h,m</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>SE/SE</td>
<td>EE</td>
<td>a,c,f,i,n</td>
<td>−</td>
<td>c,h</td>
<td>p</td>
<td>a,p</td>
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<td>−</td>
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<td>4780</td>
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<td>+</td>
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<td>+/PE</td>
<td>a,b,c</td>
<td>−</td>
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<td>+</td>
<td>+</td>
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<td>SE/SE</td>
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<td>a,b,c</td>
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<td>−</td>
<td>−</td>
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<td>37 (5)</td>
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<td>+/PE</td>
<td>a,b,c</td>
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<td>−</td>
<td>+</td>
<td>+</td>
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<td>−</td>
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<td>−</td>
<td>240</td>
<td>36 (5)</td>
<td>+</td>
<td>+</td>
<td>SE/SE</td>
<td>+/PE</td>
<td>a,b,c</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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<td>ND</td>
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<td>SE/SE</td>
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<td>a,b,c</td>
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<td>−5.6 (5.6)</td>
<td>−2.4</td>
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</table>

Abbreviations: a, Staphylococcus aureus; AO, angioedema; AS, asthma; b, Streptococcus agalactiae; c, Streptococcus beta-hemolyticus group G; d, Haemophilus influenzae; e, Haemophilus parainfluenzae; EE, episodic erythroderma; f, Pseudomonas aeruginosa; g, Klebsiella oxytoca; GER, gastroesophageal reflux; h, Escherichia coli; i, Klebsiella pneumoniae; ILC, ichthyosis linearis circumflexa; j, Stenotrophomonas maltophilia; k, Staphylococcus epidermidis; l, Enterobacter cloacae; m, S. aureus Panton-Valentine leukocidin–encoding gene; n, Proteus mirabilis; ND, no data; o, meticillin-resistant S. aureus; p, Diptheroid species; PE, patchy erythema and scaling; R, rhinoconjunctivitis; SE, scaly erythroderma; TI, trichorrhexis invaginata; U, urtica; − absent; + present.

SI conversion factor: To convert IgE to micrograms per liter, multiply by 0.0024.

† Infant indicates younger than 1 year; toddler, 1 to 3 years; preschool, 3 to 5 years; middle childhood, 6 to 11 years; young teens, 12 to 14 years; and teenagers, 15 to 17 years.

‡ Only the heels.

§ Periorally, cheeks, ankles, and genital area.

∥ Only the cheeks.

¶ Indicates improved.

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occurred, but overall their skin symptoms, except for external otitis, were very mild and confined to local eczematous lesions. Allergic symptoms are mild (patient VI:1) or absent (patient VI:2).

Intrafamilial phenotype severity variation was also evident in families III and V (Table). Patient III:1 has had strikingly more severe skin symptoms, allergies, and growth retardation than his 2 affected siblings. Siblings V:1 and V:2 differ in that patient V:2 has severe growth retardation whereas patient V:1 has more severe skin symptoms and allergies.

Novel c.1772delT Deletion With Similar Phenotype to the Homozygous Founder

SPINK5 Mutation

Patient VII:1 has had a phenotype similar to that of the homozygous patients with the Finnish founder SPINK5 mutation (Figure 1F). Her skin condition has improved with age, but erythrodermic flares occurred. She has developed diffuse alopecia and a progressive unilateral hearing loss as an adult (Table). Acitretin treatment showed no benefit.

Immature NK Cell Phenotype and Functional Defects With Elevated Serum IgG4 and IgE Levels

We studied NK cells extensively from 7 patients and discovered an immature phenotype with decreased CD27 expression (Figure 2A) and increased CD45RA (Figure 2B) and CD62L (L-selectin) (Figure 2C) expression. Cytotoxicity and degranulation of NK cells were found to be decreased compared with the cells of healthy volunteers (Figure 2D-F). However, the cytokine production was intact (Figure 2G).

Elevated serum IgG4 levels were observed in 4 of 5 patients studied (eTable 4 in the Supplement), which, to our knowledge, has not been reported previously. Phenotype analysis of T and B cells confirmed the decrease of nonswitched memory B cells or CD27+ memory B cells in NS (eTable 4 in the Supplement).14

Pneumococcal vaccination responses were below the 5th percentile for all serotypes for 3 of 4 patients (eTable 4 in the Supplement). After a second vaccination, patient I:1 mounted a good response above the 10th percentile to 7 of 9 serotypes tested and a weaker response above the 5th percentile against 2 serotypes. Patient I:1 did not produce any varicella zoster antibodies to an initial vaccination but showed a good response after a second vaccination. Diphtheria and tetanus vaccine responses were normal for patients I:1 and II:1.

Discussion

We describe herein a unique cohort with 4 different SPINK5 mutations found in 12 patients with NS, including patients from 3 multiplex families. We identified a homozygous mutation c.652C>T in exon 8 of SPINK5 in 8 patients with NS from 5 families and at a compound heterozygous state in 2 siblings from
as a sixth family. All these families originate from the same western region of Finland. The c.652C>T mutation was thus found in 10 of the 11 Finnish patients with NS studied (91%), and haplotype analysis confirmed it as a Finnish founder mutation.

More than 70 different \textit{SPINK5} mutations have been reported in patients with NS, and so far the c.891C>T (Cys297Cys) mutation in exon 11 has been the most common mutation found in families (9.5%) originating from the Mediterranean countries.\textsuperscript{4,22} We also identified a novel \textit{SPINK5} mutation in a patient from an isolated coastal region of Finland, who was homozygous for c.1772delT in exon 19. The expected prevalence of NS is 1 in 200 000, which estimates 27 patients with NS in Finland. Misdiagnosis and high neonatal mortality rates may explain why some patients with NS have not been identified.\textsuperscript{23}

Clear genotype-phenotype correlations have not been made in NS, although some mutations have been reported to be lethal early in infancy or associated with a severe phenotype.\textsuperscript{18,22,23} Genotype-phenotype correlations have been reported\textsuperscript{24} in Japanese patients with, for instance, cutaneous severity, growth retardation, and skin infection. Patients with NS who are homozygous for the \textit{SPINK5} Finnish founder mutation and the patient with the c.1772delT in exon 19 shared a comparable phenotype, with scaling and erythroderma,
Phenotype Variation and Immature Immunity in Netherton Syndrome

Original Investigation Research

Netherton syndrome has been proposed to be a primary immunodeficiency syndrome, owing to reduced memory B cells, decreased NK cell cytotoxicity, and selective antibody deficiency to bacterial antigens, such as pneumococcal polysaccharides. No detailed data on the defects of innate immune response in NS exist to date. Single cases of reduced levels of IgA, IgG2, IgG3, and NK cells have been reported. Natural killer cells operate in the innate immune response, but their role in allergic diseases is still unclear. The NK cell phenotype of our patients with NS was immature, and the cytotoxic capacity and degranulation of the NK cells were impaired. This result could be owing in part to the age difference between the adult controls and the children with NS. However, the findings were similar in patient VII:1, who was in her 50s at the time of blood sampling. Our results also indicate impaired B-cell maturation and immunodeficiency as earlier reported in 3 patients with NS. Non-switched memory B cells, levels of which were found to be low, express IgM and play a role in the immune response against encapsulated bacteria. Our patients had recurrent skin infections, conjunctivitis, and external otitis caused by encapsulated and other bacteria. Also poor initial pneumococcal vaccine responses were possibly caused by impaired B-cell maturation.

How LEKTI deficiency precisely contributes to these immune cell deficiencies is currently unknown, but LEKTI is also expressed in the oral mucosa, tonsils, and Hassall corpuscles in the thymus, all relevant for T- and B-cell maturation. Treatment with IVIG has proven beneficial in 5 patients with NS, although its precise immunomodulatory mechanisms are not understood. The overall condition of patient II:1 improved considerably with IVIG treatment. Basic NS skin treatment consists of regularly used topical emollients. Short courses of moderate-strength corticosteroid therapy or pimecrolimus or tacrolimus therapy may be used on limited skin areas for eczematous flares, but significant absorption may be a problem owing to the skin barrier defect. Oral retinoid treatment is regarded as mostly unfavorable. Anti-tumor necrosis factor (infliximab) treatment showed significant improvement in 1 adult patient.

A new finding was the significant elevation of serum IgG4 levels. Production of IgG4 is induced by Th2 cytokines and by interleukin 10, which is produced by regulatory T cells. Strong allergen exposure and allergen immunotherapy induce IgG4 antibodies that are thought to act as blocking antibodies that induce clinical tolerance. In NS, a strong allergen exposure may explain elevated IgG4 levels, although patient VIII:1 with elevated IgG4 levels had only limited allergies. The rarity of NS limits this study, and these results should be expanded in the future to include more patients with NS.

Conclusions

This work identifies a predominant founder SPINK5 mutation in Finnish patients with NS, with common clinical features and interindividual variations. The study illustrates genotype-phenotype variation and elevated IgG4 levels in NS. Functional defects of NK cells and a B-cell maturation effect have a possible role in the frequent infections seen in patients with NS.

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